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Means and methods for modulating stomata characteristica in plants

The present invention relates to recombinant DNA molecules comprising nucleic acid molecules encoding subtilisin-like serine proteases that are involved in the regulation of stomatal density in plants; wherein said nucleic acid molecules could be operably linked to regulatory elements allowing the expression of the nucleic acid molecules in plants. The present invention also provides vectors comprising said recombinant DNA molecules as well as plant cells, plant tissues and plants transformed therewith. The present invention further relates to the use of the aforementioned recombinant DNA molecules and vectors in plant cell and tissue culture, plant breeding and/or agriculture, in particular for the production of plants with improved traits.

Stomatal complexes (stomata) are specialised structures in the epidermices of all higher land plants that mediate and control the gas exchange between the internal tissues of the plants and the atmosphere. They consist of two guard cells that flank a central pore. In many plant species these central guard cells are surrounded by morphologically distinct epidermal cells (subsidiary cells). Usually, more than 90% of the gas exchange between a leaf and the atmosphere (uptake of CO_2 into the leaf and release of H_2O vapor) occurs through the stomatal pores. The major function of the stomata is to create an optimal balance between sufficient CO_2 uptake and limited water loss. To achieve this, short term control (in the range of minutes to hours) is exerted on the stomatal conductance by opening and closure of the stomatal pores through turgor driven movement of the guard cells (for review see Zeiger, Farquhar and Cowan (eds.) Stomatal Function, Stanford University Press, Stanford 1987; Willmer and Fricker (eds.) Topics in Functional Biology, 2. Stomata, Second Edition, Chapman and Hall Ltd., London, New York, 1996). Besides these rapid and transient changes, long term modulation of stomatal characteristics occurs which predominantly involve morphological aspects such as presence or absence of stomata in the upper and/or lower leaf epidermis, density of stomata in the leaf

epidermices, or the size of the stomata. These features are determined both, by endogenous (genetic) and by exogenous (environmental) factors. Hints towards genetic control were obtained through the observation of a broad variation of the stomatal density between different species of the same genus, between varieties or cultivars of the same species, or in F1 hybrids. Genetic analysis revealed multigenic, oligogenic, or monogenic control and a high heritability of characters such as stomatal density or size (for review see Jones, *In* Stomatal Function, Eds. E. Zeiger, G.D. Farquhar and I.R. Cowan, pp. 431-443, Stanford University Press, Stanford 1987). In addition to this endogenous control, stomatal characteristics are also modulated through environmental effects. Thus, air humidity (e.g. Schürmann, 1959, *Flora* 147, 417-520), light intensity (e.g. Gay and Hurd, 1975, *New Phytol.* 75, 37-46; Schoch et al., 1980, *J. Exp. Bot.* 31, 1211-1216; Rahim and Fordham, 1991, *Ann. Bot.* 67, 167-171), and CO_2-concentration (e.g. Woodward, 1987, *Nature* 327, 617-618; Woodward and Bazzaz, 1988, *J. Exp. Bot.* 39, 1771-1781; Goodfellow et al., 1997, *Tree Physiol.* 17, 291-299) were found to affect stomatal density. In several studies, stomatal density was found to be associated with plant yield (e.g. Walton, 1974, *Can J. Plant Sci.* 54, 749-754). Pima cotton varieties selected for high yield upon growth under conditions of high irradiance and artificial irrigation exhibit increased stomatal density associated with increased stomatal conductance and reduced leaf temperature (Cornish et al., 1991, *Plant Physiol.* 97, 484-489; Lu and Zeiger, 1994, *Physiol. Plant.* 92, 273-278; Lu et al., 1994, *Physiol. Plant.* 92, 266-272; Srivastava et al., 1995, *Plant Sci.* 19, 125-131). A similar relationship between stomatal conductance and yield was observed for a series of bread wheat varieties (Lu et al., 1998, *J. Exp. Bot.* 49, 453-460). According to these data, the modulation of stomatal characteristics are of high importance for the improvement of elite cultivars of crop plants. In the area of agriculture and forestry, a major aim is the continuous improvement of the crop plants with respect to higher yielding to provide sufficient food for the growing global population and to ensure the supply of renewable resources. Traditionally, progress towards higher yielding varieties is attempted through breeding, a very labour und time consuming process to be conducted separately for every relevant plant species. Some progress has already been achieved through the application of genetic engineering to plants, i.e. the introduction and expression of recombinant nucleic acid molecules in plants. Such approaches are advantageous as they can usually be applied to many different

plant species. In EP-A 0 511 979, for instance, the use of a procaryotic asparagine synthetase for expression in plant cells is described that, among other changes, leads to increased biomass production. WO 96/21737 describes yield increases in plants achieved through the expression of de- or non-regulated fructose-1,6-bisphosphatase through enhanced rate of photosynthesis. In WO 96/17069, the enhancement of biomass production in transgenic plants achieved through expression of a polyphosphate kinase from *E.coli* is described. In contrast to these cases, however, no means for a directed manipulation through genetic engineering of stomatal density or distribution in plants were hitherto available, due to the complete lack of knowlegde about genes that are involved in the control of these stomatal characteristics.

Recently, an *Arabidopsis thaliana* mutant, R-558, has been isolated after chemical mutagenesis which shows a two to four-fold increase in the stomatal density of all aerial plant organs, in the leaves in particular and the occurrence of ca. 10% clustered stomata, i.e. stomata placed in direct contact to at least one other stomata (D. Berger, 1997, PhD Thesis Freie Universität Berlin). Besides a minor change in the length of the pedicelli, no other morphological changes were visible in the mutant plants. The form and size of the leaves as well as the structure of the mesophyll (number of cell layers in palisade and spongy parenchyma, form and size of the mesophyll cells) and the intercellular system (including the substomatal cavities) are unchanged. The increased stomatal density resulted in elevated transpiration (loss of H_2O) and was associated with increased dry matter content in the leaves which in the wild type was ca. 3% and in the mutant ca. 7%. It was furthermore shown that the increased stomatal density in the R-558 mutant was associated with increased leaf fresh (+ 15%) and dry (+30%) weight, increasd glucose (+70%), fructose (+65%), and protein (+50%) contents in leaves, and enhanced transpiration and CO_2-assimilation (D. Berger, 1997, PhD Thesis, Freie Universität Berlin) in comparison to the wild type. The mutation which caused the increased stomatal density has been mapped relative to a set of (molecular) genetic markers to a ca. 0.59 cM interval located on the top arm of chromosome 1 of *Arabidopsis thaliana* (D. Berger, 1997, PhD Thesis, Freie Universität Berlin).

However, the regulation of stomatal density and distribution in plants is still not fully understood and means that can be used to manipulate stomatal characteristics such

as density and distribution that may have applications in several aspects of agriculture were hitherto not available.

Thus, the technical problem underlying the present invention was to comply with the need for means and methods for modulating the stomatal density in plants.
The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a recombinant DNA molecule comprising

(i) a nucleic acid molecule encoding a subtilisin-like serine protease or encoding a biologically active fragment of such a protein, selected from the group consisting of

(a) nucleic acid molecules comprising a nucleotide sequence encoding a protein comprising the amino acid sequence as given in SEQ ID NO: 3;

(b) nucleic acid molecules comprising a nucleotide sequence as given in SEQ ID NO: 1;

(c) nucleic acid molecules encoding a protein comprising at least the D region, H region, substrate binding site and/or S region of the subtilisin-like serine protease encoded by a nucleic acid molecule of (a) or (b); or

(d) nucleic acid molecules hybridizing with the complementary strand of a nucleic acid molecule as defined in any one of (a) to (c);

(e) nucleic acid molecules encoding a protein the amino acid sequence of which is at least 50% identical to the amino acid sequence encoded by a nucleic acid molecule of any one of (a) to (c);

(f) nucleic acid molecules, the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of a nucleic acid molecule as defined in any one of (a) to (e); or

(ii) a nucleic acid molecule encoding a mutant non-active or hyper-active form of or an antibody against the subtilisin-like serine protease encoded by a nucleic acid molecule of (i); or

(iii) a nucleic acid molecule which specifically hybridizes with a nucleic acid molecule of (i) or the complementary strand thereof.

The present invention is based on the identification of a new class of genes represented by *SDD1* from *Arabidopsis thaliana* which are involved in the control of stomatal density and distribution. The SDD1 gene is mutated in the *Arabidopsis thaliana* mutant R-558; see Examples 1 to 3. Computer-assisted amino acid sequence analysis of the protein encoded by this gene revealed that it belongs to a family of subtilisin-like serine proteases; see Example 4.

The term "subtilisin like-serine protease", as used herein, means a specific class of serine proteases, called subtilisins or dibasic processing endoproteases. In subtilisins, four regions form the catalytic triad and the substrate binding site and are most highly conserved among subtilisins; see also Example 4. In the context of the present invention, subtilisin-like serine proteases also means such proteins which show a homology of at least 50% to the sequence shown in SEQ ID NO: 3. Furthermore, the substrate binding site can have the amino acid motif VICAAGNNG. While a function of such proteins in the regulation of stomatal density in plants was hitherto unknown, the present invention for the first time provides evidence that the described nucleic acid molecules encode proteins that are involved in controlling the density and the distribution of stomata in plants. Furthermore, it is shown that plants lacking such proteins show altered morphological and physiological features of high agronomic importance.

Thus, the present invention for the first time clearly establishes that stomatal characteristics such as density and distribution can be specifically modulated through the application of genetic engineering techniques and provides extremely useful tools for example to:

(i) generate plants with increased stomatal density and consequently with enhanced CO_2 assimilation, reduced leaf temperature, enhanced leaf fresh and dry weight, and enhanced sugar and protein contents in leaves;

(ii) generate plants with decreased stomatal density and consequently with reduced water loss and thus lower water consumption;

(iii) counteract environmental changes such as raises in atmospheric CO_2 levels, temperature and irradiation that would cause changes in stomatal density to sub- or supra-optimal levels;

In general a nucleic acid molecule encoding a subtilisin-like serine protease can be derived from any material source, for example, from any organism, preferably plants possessing such molecules, preferably form monocotyledonous or dicotyledonous plants, in particular from any plant of interest in agriculture, horticulture or wood culture, such as crop plants, namely those of the family Poaceae, any starch producing plants, such as potato, maniok, leguminous plants, oil producing plants, such as oilseed rape, linenseed, etc., plants using polypeptide as storage substances, such as soybean, plants using sucrose as storage substance, such as sugar beet or sugar cane, trees, ornamental plants etc. or plants belonging to the family Gramineae.

Furthermore, nucleic acid molecules can be used in accordance with the present invention hybridizing to the above-described nucleic acid molecules and encoding subtilisin-like serine protease. Such nucleic acid molecules can be isolated, e.g., from libraries, such as cDNA or genomic libraries by techniques well known in the art. For example, hybridizing nucleic acid molecules can be identified and isolated by using the above-described nucleic acid molecules or fragments thereof or complements thereof as probes to screen libraries by hybridizing with said molecules according to standard techniques. Possible is also the isolation of such nucleic acid molecules by applying the polymerase chain reaction (PCR) using as primers oligonucleotides derived form the above-described nucleic acid molecules. Nucleic acid molecules which hybridize with any of the aforementioned nucleic acid molecules also include fragments, derivatives and allelic variants of the above-described nucleic acid molecules that encode subtilisin-like serine proteases or biologically active fragments thereof. Fragments are understood to be parts of nucleic acid molecules long enough to encode the described protein or a fragment thereof having the biological activity as defined above. Preferably, said fragment comprises at least one region of subtilisin-like serine protease as defined in section (i) (c) supra.

The term "derivative" means in this context that the nucleotide sequence of these nucleic acid molecules differs from the sequences of the above-described nucleic acid molecules in one or more nucleotide positions and are highly homologous to said nucleic acid molecules. Homology is understood to refer to a sequence identity of at least 50 %, particularly an identity of at least 70 %, preferably more than 80 %

and still more preferably more than 90 %. The deviations from the sequences of the nucleic acid molecules described above can, for example, be the result of nucleotide substitution(s), deletion(s), addition(s), insertion(s) and/or recombination(s) either alone or in combination, that may naturally occur or be produced via recombinant DNA techniques well known in the art; see for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Homology further means that the respective nucleic acid molecules or encoded proteins are functionally and/or structurally equivalent. The nucleic acid molecules that are homologous to the nucleic acid molecules described above and that are derivatives of said nucleic acid molecules are, for example, variations of said nucleic acid molecules which represent modifications having the same biological function, in particular encoding proteins with the same or substantially the same biological activity as defined herein. They may be naturally occurring variations, such as subtilisin-like serine protease encoding sequences from other prokaryotes and eukaryotes, respectively, or mutations. These mutations may occur naturally or may be obtained by mutagenesis techniques, see supra. The allelic variations may be naturally occurring allelic variants as well as synthetically produced or genetically engineered variants; see supra. For example, the amino acid sequences of plant subtilisin-like serine proteases share significant similarities with bacterial, yeast and mammalian subtilisin-like serine protease; see Example 4. In addition, nucleic acid molecules can be employed in accordance with the present invention that encode homologs or analogs of the above described subtilisin-like serine protease but where otherwise unrelated to those proteins. Such proteins that do not display significant homologies to common subtilisin-like serine protease can be identified by a person skilled in the art using techniques well known in the art, for example, via complementation of mutant genes, for example, in corresponding mutant plants; see Example 3.

The proteins encoded by the various derivatives, variants, homologs or analogs of the above-described nucleic acid molecules may share specific common characteristics, such as molecular weight, immunological reactivity, conformation, etc., as well as physical properties, such as electrophoretic mobility, chromatographic behavior, sedimentation coefficients, pH optimum, temperature optimum, stability, solubility, spectroscopic properties, etc. All these nucleic acid

molecules and derivatives can be employed in accordance with the present invention as long as the biological activity of the encoded protein remains substantially unaffected in kind, namely that the protein is capable of modulating stomata density in plants. Any one of the above described nucleic acid molecules, in particular those that represent hyper-active mutant forms of subtilisin-like serine proteases are particular suitable for overexpression in transgenic plants. These transgenic plants may either possess an endogenous functional subtilisin-like serine protease or they may lack the corresponding genes, e.g. due to mutation.

The nucleic acid molecules mentioned in section (ii) and (iii) are particular useful for the suppression of genes encoding subtilisin-like serine proteases in plants. Hence, in one embodiment said nucleic acid molecules are preferably of at least 15 nucleotides in length hybridizing specifically with a nucleic acid molecule as described above or with a complementary strand thereof. Specific hybridization occurs preferably under stringent conditions and implies no or very little cross-hybridization with nucleotide sequences encoding no or substantially different proteins. In particular stringent conditions mean e.g. the use of an aqueous solution of 1% BSA, 1mM EDTA, 0.5 M $NaHPO_4$ pH7.2, 7% SDS and incubation at 65°C. Such nucleic acid molecules may be used as probes and/or for the control of gene expression. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary in length. Preferred are nucleic acid probes of 16 to 35 nucleotides in length. Of course, it may also be appropriate to use nucleic acids of up to 100 and more nucleotides in length. The nucleic acid probes of the invention are useful for various applications. On the one hand, they may be used as PCR primers for amplification of nucleic acid sequences according to the invention. Another application is the use as a hybridization probe to identify nucleic acid molecules hybridizing with a nucleic acid molecule of the invention by homology screening of genomic DNA or cDNA libraries. Nucleic acid molecules according to this preferred embodiment of the invention which are complementary to a nucleic acid molecule as described above can be used for repression of expression of a subtilisin-like serine protease encoding gene, for example due to an antisense or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-B1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a nucleic acid

molecule of the invention or part thereof. Selection of appropriate target sites and corresponding ribozymes can be done as described, for example, in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460. Furthermore, the person skilled in the art is well aware that it is also possible to label such a nucleic acid probe with an appropriate marker for specific applications, such as for the detection of the presence of a nucleic acid molecule of the invention in a sample derived from an organism, in particular plants. The above described nucleic acid molecules may either be DNA or RNA or a hybrid thereof. Furthermore, said nucleic acid molecule may contain, for example, thioester bonds and/or nucleotide analogues, commonly used in oligonucleotide anti-sense approaches. Said modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in the cell.

Furthermore, nucleic acid molecules encoding antibodies specifically recognizing a subtilisin-like serine protease or parts, i.e. specific fragments or epitopes, of such a protein can be used for inhibiting the activity of the protein in plants. These antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned peptides can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. These antibodies can be used, for example, for the immunoprecipitation and immunolocalization of proteins according to the invention as well as for the monitoring of the synthesis of such proteins, for example, in recombinant organisms, and for the identification of compounds interacting with the protein according to the invention. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies selections, yielding a high increment of affinity from a single library of phage antibodies which bind to an epitope of the protein of the invention (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13). Expression of antibodies or antibody-like molecules in plants can be achieved

by methods well known in the art, for example, full-size antibodies (Düring, Plant. Mol. Biol. 15 (1990), 281-293; Hiatt, Nature 342 (1989), 469-470; Voss, Mol. Breeding 1 (1995), 39-50), Fab-fragments (De Neve, Transgenic Res. 2 (1993), 227-237), scFvs (Owen, Bio/Technology 10 (1992), 790-794; Zimmermann, Mol. Breeding 4 (1998), 369-379;Tavladoraki, Nature 366 (1993), 469-472) and dAbs (Benvenuto, Plant Mol. Biol. 17 (1991), 865-874) have been successfully expressed in Tobacco, Potato (Schouten, FEBS Lett. 415 (1997), 235-241) or *Arabidopsis*, reaching expression levels as high as 6.8% of the total protein (Fiedler, Immunotechnology 3 (1997), 205-216).

In addition, nucleic acid molecules encoding mutant forms of a subtilisin-like serine protease can be used to interfere with the activity of the wild type protein. Such mutant forms preferably have lost their biological activity as defined above and may be derived from the corresponding subtilisin-like serine protease by way of amino acid deletion(s), substitution(s), and/or additions in the amino acid sequence of the protein. As mentioned above, mutant forms of subtilisin-like serine proteases also encompass hyper-active mutant forms of such proteins which display, e.g. an increased substrate affinity and/or higher substrate turnover of the same. Furthermore, such hyper-active forms may be more stable in the cell due to the incorporation of amino acids that stabilize proteins in the cellular environment. These mutant forms may be naturally occurring or genetically engineered mutants, see also supra.

The recombinant DNA molecule of the invention preferably comprises regulatory sequences allowing for the expression the nucleic acid molecules in plants. Preferably, said regulatory elements comprise a promoter active in plant cells. Expression comprises transcription of the nucleic acid molecule preferably into a translatable mRNA. Regulatory elements ensuring expression in plant cells are well known to those skilled in the art.

These regulatory elements may be homologous or preferably heterologous with respect to the nucleic acid molecule to be expressed and/or with respect to the plant species to be transformed. For example, a preferred regulatory element that can be employed in accordance with the present invention is the SDD1 promoter region as depicted in SEQ ID NO: 5 or part thereof. In general, such regulatory elements comprise a promoter active in plant cells. To obtain expression in all tissues of a

transgenic plant, preferably constitutive promoters are used, such as the 35S promoter of CaMV (Odell, Nature 313 (1985), 810-812) or promoters of the polyubiquitin genes of maize (Christensen, Plant Mol. Biol. 18 (1982), 675-689). In order to achieve expression in specific tissues of a transgenic plant it is possible to use tissue specific promoters (see, e.g., Stockhaus, EMBO J. 8 (1989), 2245-2251). Known are also promoters which are specifically active in tubers of potatoes or in seeds of different plants species, such as maize, Vicia, wheat, barley etc. Inducible promoters may be used in order to be able to exactly control expression. An example for inducible promoters are the promoters of genes encoding heat shock proteins. Also microspore-specific regulatory elements and their uses have been described (WO96/16182). Furthermore, the chemically inducible Tet-system may be employed (Gatz, Mol. Gen. Genet. 227 (1991); 229-237). Further suitable promoters are known to the person skilled in the art and are described, e.g., in Ward (Plant Mol. Biol. 22 (1993), 361-366). The regulatory elements may further comprise transcriptional and/or translational enhancers functional in plants cells. A plant translational enhancer often used is, e.g., the CaMV omega sequences and/or the inclusion of an intron (Intron-1 from the Shrunken gene of maize, for example) that has been shown to increase expression levels by up to 100-fold. (Maiti, Transgenic Research 6 (1997), 143-156; Ni, Plant Journal 7 (1995), 661-676). Furthermore, the regulatory elements may include transcription termination signals, such as a poly-A signal, which lead to the addition of a poly A tail to the transcript which may improve its stability. The termination signals usually employed are from the Nopaline Synthase gene or from the CaMV 35S RNA gene.

In a preferred embodiment of the recombinant DNA molecule of the invention, the subtilisin-like serine protease is derived from plants. Preferably, said plants are monocotyledonous or dicotyledonous plants such as those mentioned hereinbefore. A particular preferred embodiment of said plant is Arabidopsis.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages and other vectors used conventionally in genetic engineering that contain at least one recombinant DNA molecule according to the invention. Methods which are well known to those skilled in the art can be used to construct various plasmids and vectors; see, for example, the techniques described

in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the recombinant DNA molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

Advantageously the above-described vectors of the invention comprises a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hpt, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338). Useful scorable marker are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and organisms containing a vector of the invention. As described above, various selectable markers can be employed in accordance with the present invention. Advantageously, selectable markers may be used that are suitable for direct selection of transformed plants, for example, the phophinothricin-N-acetyltransferase gene the gene product of which detoxifies the herbicide L-phosphinothricin (glufosinate or BASTA); see, e.g., De Block, EMBO J. 6 (1987), 2513-2518 and Dröge, Planta 187 (1992), 142-151.

The present invention, also relates to host cells comprising a recombinant DNA molecule or vector of the invention. Host cells include prokaryotic and eukaryotic cells such as E. coli and yeast, respectively.

The recombinant DNA molecules according to the invention are in particular useful for the genetic manipulation of plant cells, plant tissue and plants in order to obtain plants with modified, preferably with improved or useful phenotypes as described above. Thus, the present invention relates to a method for the production of transgenic plants with altered stomata characteristica compared to wild type plants comprising the introduction of a recombinant DNA molecule of the invention into the genome of a plant, plant cell or plant tissue.

Methods for the introduction of foreign DNA into plants as well as the selection and regeneration of transgenic plants from plant cells and plant tissue are also well known in the art. These include, for example, the transformation of plant cells, plant tissue or plants with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, biolistic methods like particle bombardment and other methods known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow for stable integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Suitable strains of Agrobacterium tumefaciens and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of Agrobacterium tumefaciens is preferred in the method of the invention, other Agrobacterium strains, such as Agrobacterium rhizogenes, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou (1996) Trends in Plant Science 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995).

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc. Transgenic plant tissue and plants can be regenerated by methods well known in the art. There are various references in the relevant literature dealing specifically with the transformation of maize (cf. e.g. WO95/06128, EP 0 513 849; EP 0 465 875). In EP 292 435 a method is described by means of which fertile plants may be obtained starting from mucousless, friable granulous maize callus. In this context it was furthermore observed by Shillito et al., Bio/Technology 7 (1989), 581 that for regenerating fertile plants it is necessary to start from callus-suspension cultures from which a culture of dividing protoplasts can be produced which is capable to regenerate to plants. After an in vitro cultivation period of 7 to 8 months Shillito et al.

obtain plants with viable descendants which, however, exhibited abnormalities in morphology and reproductivity.

Prioli and Söndahl, Bio/Technology 7 (1989), 589 have described how to regenerate and to obtain fertile plants from maize protoplasts of the Cateto maize inbreed Cat 100-1. The authors assume that the regeneration of protoplasts to fertile plants depends on a number of various factors such as the genotype, the physiological state of the donor-cell and the cultivation conditions. With regard to rice various transformation methods can be applied, e.g. the transformation by agrobacterium-medicated gene transfer (Hiei et al., Plant J. 6 (1994), 271-282; Hiei et al., Plant Mol. Biol. 35 (1997), 205-218; Park et al., J. Plant Biol. 38 (1995), 365-371), protoplast transformation (Datta in "Gene transfer to plants", I. Potrykus, G. Spangenberg (Eds.), Springer-Verlag Berlin Heidelberg (1995), pages 66-75; Datta et al., Plant Mol. Biol. 20 (1992), 619-629; Sadasivam et al., Plant Cell Rep. (1994), 394-396), the biolistic approach (Li et al., Plant Cell Rep. 12 (1993), 250-255; Cao et al., Plant Cell Rep. 11 (1992), 586-591; Christou, Plant Mol. Biol. (1997), 197-203) and electroporation (Xu et al., in "Gene transfer to plants", I. Potrykus, G. Spangenberg (Eds.), Springer-Verlag Berlin Heidelberg (1995), pages 201-208.

Once the introduced DNA has been integrated in the genome of the plant cell, it usually continues to be stable there and also remains with the descendants of the originally transformed cell. It usually contains a selectable marker which confers resistance against biozides or against an antibiotic such as kanamycin, G 418, bleomycin, hygromycin or phosphinotricine etc. to the transformed plant cells. The individually selected marker should therefore allow for a selection of transformed cells against cells lacking the introduced DNA.

The transformed cells grow in the usual way within the plant (see also McCormick et al., Plant Cell Rep. 5 (1986), 81-84). The resulting plants can be cultivated in the usual way and cross-bred with plants having the same transformed genetic heritage or another genetic heritage. The resulting hybrid individuals have the corresponding phenotypic properties.

Two or more generations should be grown in order to ensure whether the phenotypic feature is kept stably and whether it is transferred. Furthermore, seeds should be

harvested in order to ensure that the corresponding phenotype or other properties will remain.

In general, the plants, plant cells and plant tissue which can be modified with a recombinant DNA molecule or vector according to the invention can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, peanut, soybean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

Thus, the present invention relates also to transgenic plant cells which contain a nucleic acid molecule as defined above or a recombinant DNA molecule or vector according to the invention wherein the nucleic acid molecule is foreign to the transgenic plant cell.

By "foreign" it is meant that the nucleic acid molecule is either heterologous with respect to the plant cell, this means derived from a cell or organism with a different genomic background, or is homologous with respect to the plant cell but located in a different genomic environment than the naturally occurring counterpart of said nucleic acid molecule. This means that, if the nucleic acid molecule is homologous with respect to the plant cell, it is not located in its natural location in the genome of said plant cell when stably integrated into the genome, in particular it is surrounded by different genes. In this case the nucleic acid molecule may be either under the control of its own promoter or under the control of a heterologous promoter. The nucleic acid molecule, vector or recombinant DNA molecule according to the invention which is present in the plant cell may either be integrated into the genome of the plant cell or it may be maintained in some form extrachromosomally.

In one aspect the present invention relates to a transgenic plant cell comprising stably integrated into the genome a recombinant DNA molecule of the invention or a vector of the present invention or obtainable according to the method of the invention wherein the expression of the nucleic acid molecule results in an increased expression or activity of subtilisin-like serine proteases in transgenic plants compared to wild-type

plants. Alternatively, a plant cell having a nucleic acid molecule encoding a subtilisin-like serine protease present in its genome can be used and modified such that said plant cell expresses the endogenous gene corresponding to this nucleic acid molecules under the control of heterologous promoter and/or enhancer elements. The introduction of the heterologous promoter and mentioned elements which do not naturally control the expression of a nucleic acid molecule encoding a subtilisin-like serine protease using, e.g., gene targeting vectors can be done according to standard methods, see supra and, e.g., Hayashi, Science 258 (1992), 1350-1353; Fritze and Walden, Gene activation by T-DNA tagging. In *Methods in Molecular* biology 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, Physiologia Plantarum 78 (1990), 105-115). Suitable promoters and other regulatory elements such as enhancers include those mentioned hereinbefore.

Furthermore, the present invention relates to transgenic plants or plant tissue comprising plant cells of the invention or obtainable by the above described method Preferably, the transgenic plant of the invention displays a decreased stomata density, lower conductance of stomata and/or the water consumption is lowered compared to wild type plants.

In another aspect, the present invention relates to a transgenic plant cell which contains stably integrated into the genome a recombinant DNA molecule of the invention or part thereof, a vector of the present invention or obtainable according to the method of the invention, wherein the presence, transcription and/or expression of the nucleic acid molecule or part thereof leads to reduction of the synthesis or the activity of subtilisin-like serine proteases in transgenic plants compared to wild type plants. Preferably, said reduction is achieved by an antisense, sense, ribozyme, co-suppression in vivo mutagenesis and/or dominant mutant effect. Therefore, DNA molecules encoding an antisense RNA which is complementary to transcripts of a DNA molecule of the invention are also the subject matter of the present invention, as well as these antisense molecules. Thereby, complementarity does not signify that the encoded RNA has to be 100% complementary. A low degree of complementarity is sufficient, as long as it is high enough in order to inhibit the expression of a protein of the invention upon expression in plant cells. The transcribed RNA is preferably at least

90% and most preferably at least 95% complementary to the transcript of the nucleic acid molecule of the invention. In order to cause an antisense-effect during the transcription in plant cells such DNA molecules have a length of at least 15 bp, preferably a length of more than 100 bp and most preferably a length or more than 500 bp, however, usually less than 5000 bp, preferably shorter than 2500 bp.

The invention further relates to DNA molecules which, during expression in plant cells, lead to the synthesis of an RNA which in the plant cells due to a cosupression-effect reduces the expression of the nucleic acid molecules of the invention encoding the described protein. The invention also relates to RNA molecules encoded thereby. The principle of the cosupression as well as the production of corresponding DNA sequences is precisely described, for example, in WO90/ 12084. Such DNA molecules preferably encode an RNA having a high degree of homology to transcripts of the nucleic acid molecules of the invention. It is, however, not absolutely necessary that the coding RNA is translatable into a protein.

In a further embodiment the present invention relates to DNA molecules encoding an RNA molecule with ribozyme activity which specifically cleaves transcripts of a DNA molecule of the invention as well as these encoded RNA molecules.
Ribozymes are catalytically active RNA molecules capable of cleaving RNA molecules and specific target sequences. By means of recombinant DNA techniques it is possible to alter the specificity of ribozymes. There are various classes of ribozymes. For practical applications aiming at the specific cleavage of the transcript of a certain gene, use is preferably made of representatives of two different groups of ribozymes. The first group is made up of ribozymes which belong to the group I intron ribozyme type. The second group consists of ribozymes which as a characteristic structural feature exhibit the so-called "hammerhead" motif. The specific recognition of the target RNA molecule may be modified by altering the sequences flanking this motif. By base pairing with sequences in the target molecule these sequences determine the position at which the catalytic reaction and therefore the cleavage of the target molecule takes place. Since the sequence requirements for an efficient cleavage are low, it is in principle possible to develop specific ribozymes for practically each desired RNA molecule.

In order to produce DNA molecules encoding a ribozyme which specifically cleaves transcripts of a DNA molecule of the invention, for example a DNA sequence encoding a catalytic domain of a ribozyme is bilaterally linked with DNA sequences which are homologous to sequences encoding the target protein. Sequences encoding the catalytic domain may for example be the catalytic domain of the satellite DNA of the SCMo virus (Davies et al., Virology 177 (1990), 216-224 and Steinecke et al., EMBO J. 11 (1992), 1525-1530) or that of the satellite DNA of the TobR virus (Haseloff and Gerlach, Nature 334 (1988), 585-591). The DNA sequences flanking the catalytic domain are preferably derived from the above-described DNA molecules of the invention. The expression of ribozymes in order to decrease the activity in certain proteins in cells is also known to the person skilled in the art and is, for example, described in EP-B1 0 321 201. The expression of ribozymes in plant cells was, for example, described, in Feyter et al. (Mol. Gen. Genet. 250 (1996), 329-338).

In a preferred embodiment this reduction is effected by means of an antisense effect. For this purpose the DNA molecules of the invention or parts thereof are linked in antisense orientation with a promoter ensuring the transcription in plant cells and possibly with a termination signal ensuring the termination of the transcription as well as the polyadenylation of the transcript. In order to ensure an efficient antisense effect in the plant cells the synthesized antisense RNA should exhibit a minimum length of 15 nucleotides, preferably of at least 100 nucleotides and most preferably of at least 500 nucleotides. Furthermore, the DNA sequence encoding the antisense RNA should be homologous with respect to the plant species to be transformed. However, DNA sequences exhibiting a high degree of homology to DNA sequences which are present in the cells in endogenic form may also be used, preferably with a homology of more than 95%.

In a further embodiment the reduction of the amount of proteins encoded by the DNA molecules of the invention is effected by a ribozyme effect. The basic effect of ribozymes as well as the construction of DNA molecules encoding such RNA molecules have already been described above. In order to express an RNA with ribozyme activity in transgenic cells the above-described DNA molecules encoding a riboyzme are linked with DNA elements which ensure the transcription in plant cells, particularly with a promoter and a termination signal. The ribozymes synthesized in the

plant cells lead to the cleavage of the mRNA encoding the subtilisin-like serine proteases described above.

Alternatively, the subtilisin-like serine protease activity in the plant cells of the invention can also be decreased by a co-suppression effect. The method is known to the person skilled in the art and is, for example, described in Jorgensen Jorgensen, Trends Biotechnol. 8 (1990), 340-344; Niebel et al. (Curr. Top. Microbiol. Immunol. 197 (1995), 91-103; Flavell et al. (Curr. Top. Microbiol. Immunol. 197 (1995), 43-36; Palaqui and Vaucheret, Plant. Mol. Biol. 29 (1995), 149-159; Vaucheret et al., Mol. Gen. Genet. 248 (1995), 311-317; de Borne et al., Mol. Gen. Genet. 243 (1994), 613-621 and in other sources.

Furthermore, the subtilisin-like serine protease activity in the plant cells of the invention can also be decreased by the so-called "in vivo mutagenesis", for which a hybrid RNA-DNA oligonucleotide ("chimeroplast") is introduced into cells by transformation of cells (Kipp et al., poster session at the 5th International Congress of Plant Molecular Biology, September 21-27, 1997, Singapore; Dixon and Arntzen, meeting report on "Metabolic Engineering in Transgenic Plants", Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15 (1997), 441-447; WO95/15972; Kren et al., Hepatology 25 (1997), 1462-1468; Cole-Strauss et al., Science 273 (1996), 1386-1389).
Part of the DNA component of the RNA-DNA oligonucleotide is homologous to a nucleic acid sequence of an endogenous subtilisin-like serine protease, in comparison to the nucleic acid sequence of the endogenous subtilisin-like serine protease it displays, however, a mutation or contains a heterologous region which is surrounded by the homologous regions. By means of base pairing of the homologous regions of the RNA-DNA oligonucleotide and of the endogenous nucleic acid molecule followed by a homologous recombination the mutation contained in the DNA component of the RNA-DNA oligonucleotide or the heterologous region can be transferred to the genome of a plant cell. This results in a decrease of the activity.

In addition, the present invention relates to transgenic plants or plant tissue comprising the above described plant cells of the invention.

In a preferred embodiment the transgenic plant displays increased stomatal density, higher conductance of stomata and/or higher content of sugars and protein in plant leaves compared to wild type plants. An increase in the stomatal density is understood to refer to an elevated content of stomata in all aerial plant organs, preferably in the leaves of plants of the present invention in the order of at least about 10% compared to the corresponding non-transformed wild type plant, which already provides for beneficial effects on the vitality of the plant such as, e.g., improved dry matter. Advantageously, the stomatal density is increased by at least about 50%, preferably by more than about 75%, particularly preferred at least about more than 100% and still more preferably more than about 200%. With respect to a decrease in the stomatal density due to the increased expression or activity of subtilisin-like serine proteases in the transgenic plant of the invention the reciprocal definition mutatis mutandis applies.

In yet another aspect, the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which contain transgenic plant cells described above, i.e. at least one recombinant DNA molecule or vector according to the invention and/or which are derived from the above described plants. Harvestable parts can be in principle any useful parts of a plant, for example, leaves, stems, flowers, fruit, seeds, roots etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks etc.

In addition, the present invention relates to a kit comprising the recombinant gene or the vector of the invention. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic plant cells, plant tissue or plants. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art. The kit or its ingredients according to the invention can be used in plant cell and plant tissue culture, for example in agriculture. The kit of the invention and its

ingredients are expected to be very useful in breeding new varieties of, for example, plants which display improved properties such as those described herein.

Furthermore, the present invention relates to use of at least one nucleic acid molecule encoding and/or regulating the expression of a subtilisin-like serine protease, a nucleic acid molecule hybridizing with such a nucleic acid molecule, a nucleic acid molecule encoding a product that interferes with the expression or activity of subtilisin-like serine proteases in plants, or a recombinant DNA molecule or vector of the invention in the production of transgenic plants for increasing yield, and/or increasing stomatal density, and/or increasing leaf fresh and/or dry weight, and/or increasing leaf dry matter content, and/or increasing sugar content in leaves, and/or increasing protein content in leaves, and/or increasing CO_2-assimilation, and/or sustaining photosynthesis (prevention of photoinhibition) under conditions of high irradiance (see Example 1), and/or changing the water consumption of plants, and or couteracting the consequences of changing environmental contitions with respect to stomatal density by the inhibition or stimulation of a subtilisin-like serine protease encoding gene. Preferably such nucleic acid molecules are derived from plant genes encoding subtilases. Modulation of the activity of these genes leads to several morphological and physiological changes that are useful for the engineering of improved plants for agriculture, wood culture, or horticulture. Furthermore, the above described nucleic acid molecules and the recombinant DNA molecules and vectors according to the invention may be useful for the alteration or modification of plant/pathogene interaction. The term "pathogen" includes, for example, bacteria, viruses and fungi as well as protozoa. The plants, plant tissue and plant cells of the invention as well as harvestable parts and propagation of such plants can be used for the preparation of feed and food or additives therefor.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and

addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The Figures show:

Figure 1: Top: Release of water vapour (transpiration) from leaves of the wildtype (wt) and the R-558 mutant (R-558) at irradiances of 300 µE (µmol m^{-2} s^{-1}) and 1200 µE (µmol m^{-2} s^{-1}) of white light measured by an infrared gas analyzer.
Bottom: Net uptake of CO_2 (assimilation) into leaves of the wildtype and the R-558 mutant (R-558) at irradiances of 300 µE (µmol m^{-2} s^{-1}) and 1200 µE (µmol m^{-2} s^{-1}) of white light measured by an infrared gas analyzer.

Figure 2: Schematic representation of the SDD1 protein marked with the amino acid positions bordering the putative pre- and the pro-sequence and the positions of the four invariant amino acids (D, H, N, S) found in all known subtilisins. Furthermore, the consequence of the mutation present in the R-558 mutant is indicated which converts the R codon at amino acid position 492 into a stop codon leading to the formation of a C-terminally truncated protein lacking the essential serine residue at position 552 (S552).

Figure 3: Schematic representation of the plasmid pG-*SDD1*

Figure 4: Schematic representation of the plasmid p35S-*SDD1*

Figure 5: Sequence alignments in the four highly conserved domains, the D region, the H region, the substrate binding site, and the S region of the subtilisins Subtilisin BPN´ (Wells et al. 1983, Nucleic Acids Res. 11,

7911-7925), the KEX2 of yeast (Mizuno et al. 1988, *Biochem. Biophys. Res. Commun.* 156, 246-254), the human FURIN/PACE (Wise et al. 1990, *Proc. Natl. Acad. Sci USA* 87, 9378 - 9382), the human PC1/PC3 (Seidah et al., 1991, *Mol. Endocrinol.* 5, 111 - 122; Smeekens et al., 1991, *Proc. Natl. Acad. Sci. USA* 88, 340 - 344), the CUCUMISIN from *Cucumis melo* (Yamagata et al., 1994, *J. Biol. Chem.* 269, 32725 - 32731), LeP69 from *Lycopersicon esculentum* (Tornero et al., 1996, *Proc. Natl. Acad. Sci. USA* 93, 6332 - 6337), the AG12 from *Alnus glutinosa* (Ribeiro et al., 1995, *Plant Cell* 7, 785 - 794) and of SDD1. The positions of the invariant amino acids are marked with *. Identical amino acids present at corresponding positions in the different subtilisins are highlighted with black boxes.

Figure 6: Schematic representation of the plasmid p35S-α*SDD1*

The Examples illustrate the invention:

Example 1: H_2O transpiration and CO_2 assimilation are increased in the *Arabidopsis thaliana* R-558 mutant particularly under conditions of high irradiance

Arabidopsis thaliana R-558 mutant plants and corresponding wildtype plants (wt) were grown until bolting in soil (Einheitserde Typ P / Einheitserde Typ T / sand: 2 / 1 / 1) under standard culture conditions in a climatised growth chamber at 16 h photoperiod (180 µmol m^{-2} s^{-1} fluorescent light; lamp type: TLD36W/840 and TLD36W/830, Philips, Hamburg, Germany) with day and night temperature and relative humidity of 20°C, 60% relative humidity and 16°C, 75% relative humidity, respectively. Single leaves (n=10) were clamped into a gas exchange measurement chamber of an infrared gas analyzer (Walz, Effeltrich, Germany) with H_2O release from as well as CO_2 uptake into the leaves were measured according to the procedure described by Muschak et al. (*Photosynthetica* 33, 455-465, 1997). As shown in Figure 1, the leaves of the mutant plants showed increased transpiration of H_2O and increased assimilation (net uptake) of CO_2 under low light (300 µmol m^{-2} s^{-}

^{1}) and under high light (1200 µmol m^{-2} s^{-1}) conditions applied during the measurements. Enhancement of CO_2 assimilation, being almost double in the R-558 mutant in comparison to the wild type, was most prominent under the high light conditions which caused a depression of CO_2 assimilation in the wild type in comparison to the low light conditions (photoinhibition).

Example 2: Isolation of the *SDD1* gene through map-based gene cloning

The genetic locus affected by the mutation in the R-558 mutant has previously been mapped to the top arm of the *Arabidopsis thaliana* chromosome 1 to an interval of approximately 0.59 cM bordered by the molecular markers IGF-20G19LE and IGF-25I3RE (D. Berger, 1997, PhD Thesis Freie Universität Berlin). Two clones of the *Arabidopsis thaliana* genomic IGF-BAC library (Mozo et al., 1998, *Mol. Gen. Genet.* 258, 562-570), IGF20D22 and IGF21M11, which fully cover this region, were sequenced by the SPP consortium (see http://sequence-www.stanford.edu/ara/SPP.html) as part of the *Arabidopsis* genome initiative (Bevan et al., 1997, *Plant Cell* 9, 476-478; http://genome-www.stanford.edu/Arabidopsis/agi.html). The 0.59 cM region was thus identified to cover 113 kb of genomic DNA sequence. In order to identify the *SDD1* gene corresponding to the mutant locus, this region was scanned for mutations by application of the restriction SSCP- (single strand conformational polymorphism) technique (Dean and Gerrard, 1991, *BioTechniques* 10, 332 - 333; Iwahana et al., 1992, *BioTechniques* 12, 64 - 66) which for this purpose was adapted for the use in plants. This approach is novel and has not been applied for mutation scanning in plants before. Thus, 57 DNA fragments of 2-kb each, were separately PCR amplified from total DNA of wild type and R-558 plants and after degestion with Alu I and/or Hinf I they were analysed through polyacrylamid gel electrophoresis as described by Dean and Gerrard, 1991 (*BioTechniques* 10, 332 – 333) and Iwahana et al., 1992 (*BioTechniques* 12, 64 – 66). A single SSCP was detected that discriminated between the two genotypes and which upon sequencing of the corresponding DNA fragments was shown to be caused by a single C/G -> T/A mutation (Seq. ID No. 1; Seq. ID No. 2). This mutation introduced a premature stop

codon into an ORF of a predicted gene spanning 2328 bp that encoded for a deduced polypeptide of 775 amino acids (Fig. 2; Seq. ID No. 3; Seq. ID No. 4; Genbank Accession AC002411; http://pgec-genome.pw.usda.gov/F20D22.anno.html#anchor12).

Example 3: Genetic complementation of the R-558 mutant by *Agrobacterium tumefaciens* – mediated DNA-transfer

In order to confirm the identity of the 2328 bp DNA sequence (Seq. ID No. 1;) as the protein coding region of the *SDD1* gene defective in the R-558 mutant, genetic complementation experiments were performed with the introduction of a wild type DNA-copy into the R-558 mutant through *Agrobacterium tumefaciens* – mediated genetic transformation.

Two plasmids were generated for this purpose:

Plasmid pG-*SDD1'* (Fig. 3) carries the 7067 bp SalI - EcoRV subfragment of the BAC IGF20D22 that includes the 2328 bp coding region of *SDD1* in addition to 2 kb upstream DNA (promotor) and 2.8 kb downstream DNA was inserted into the SalI and SmaI sites of the T-DNA vector pBIB-Hyg (Becker, 1990, *Nucleic Acids Res.* 18, 203).

The second plasmid, p35S-*SDD1* (Fig. 4), harbours the three fragments, A, B, C, inserted into the pBIB-Hyg vector (Becker, 1990, *Nucleic Acids Res.* 18, 203). Fragment A, which was inserted between the EcoRI and SacI restricition sites in the polylinker of pBIB-Hyg, includes the 35S promoter of the Cauliflower Mosaic Virus (CaMV) comprising the nucleotides 7146 through 7464 as described by Gardner et al. (*Nucleic Acids Res.* 9, 2871-2888, 1981). Fragment C contains the polyadenylation signal of the gene 3 from the T-DNA of the Ti-plasmid pTi ACH 5 (Gielen et al., *EMBO J.* 3, 835 – 846, 1984), nucleotides 11749 through 11939 which was isolated as PvuII - HindIII fragment from the plasmid pAGV 40 (Herrera-Estrella et al., *Nature* 303, 209 – 213, 1983) and which, after addition of a SphI linker to the PvuII restriction site, was inserted into the SphI and HindIII restriction sites of pBIB-Hyg. The resulting intervening plasmid was called pBIN-AR-Hyg. Fragment B covers the 2328 nucleotides coding region of the *SDD1* gene (Seq. ID

No. 1) that was amplified by PCR from the BAC IGF20D22 and provided with Asp718 and XbaI linker sequences and which was inserted into the Asp718 and XbaI restriction sites of pBIN-AR-Hyg.

Both plasmids were separately introduced into *Agrobacterium tumefaciens* according to the procedure described by Höfgen and Willmitzer (*Nucleic Acids Res.* 16, 9877, 1988) and the corresponding T-DNAs were stably introduced into the R-558 mutant by *Agrobacterium tumefaciens - in planta* transformation following the method described by Bechtold et al. (*Compt. Rend. Acad. Sci.* 316, 1194 – 1199, 1993). Transformed seedlings selected for antibiotic (Hygromycin) resistance were grown to maturity and tested for the expression of mutant or wildtype phenotypes by microscopic examination of rosette leaves cleased with 80 % ethanol.

Table 1: Analysis of stomatal density and distribution in the abaxial epidermices of cotyledons and leaves of wild type (wt), mutant (R-558) and transgenic mutant (R-558 / G-*SDD1*; R-558 / 35S-*SDD1*) plants.

	Cotyledon			Primary Leaf			
Plant	Single Stomata[a]	Clustered Stomata[b]	n[c]	Single Stomata[a]	Clustered Stomata[b]	n[c]	Density [no./mm²]
wt #1	100 %	0 %	58	100 %	0 %	163	97.0
wt #2	100 %	0 %	40	100 %	0 %	174	124.3
wt #3	100 %	0 %	51	98.9 %	1.1 %	176	125.7
wt #4	100 %	0 %	49	100 %	0 %	160	114.3
wt #5	100 %	0 %	58	100 %	0 %	166	118.6
R-558 #1	61 %	39 %	136	86 %	14 %	492	351.4
R-558 #2	56 %	44 %	62	90.1 %	9.9 %	421	300.7
R-558 #3	54 %	46 %	137	89.6 %	10.4 %	395	282.1
R-558 #4	58 %	42 %	109	93.9 %	6.1 %	409	292.1
R-558 #5	60 %	40 %	85	91.6 %	8.4 %	403	287.9
R-558 / G-*SDD1* #1	100 %	0 %	47	100 %	0 %	279	199.3
R-558 / G-*SDD1* #2	100 %	0 %	53	83.4 %	16.6 %	181	161.6
R-558 / G-*SDD1* #3	100 %	0 %	52	100 %	0 %	139	99.3
R-558 / G-*SDD1* #4	96.4 %	3.6 %	55	100 %	0 %	195	139.3
R-558 / G-*SDD1* #5	100 %	0 %	53	100 %	0 %	180	128.6
R-558 / G-*SDD1* #6	100 %	0 %	55	100 %	0 %	169	120.7
R-558 / G-*SDD1* #7	100 %	0 %	53	100 %	0 %	163	116.4
R-558 / G-*SDD1* #8	96.8 %	3.2 %	62	100 %	0 %	285	203.6
R-558 / G-*SDD1* #9	100 %	0 %	37	100 %	0 %	98	70.0
R-558 / G-*SDD1* #10	96.7 %	3.3 %	60	100 %	0 %	199	142.1
R-558 / 35S-*SDD1* #1	82 %	18 %	66	100 %	0 %	167	119.3
R-558 / 35S-*SDD1* #2	100 %	0 %	34	100 %	0 %	123	87.9
R-558 / 35S-*SDD1* #3	93 %	7 %	55	100 %	0 %	168	120
R-558 / 35S-*SDD1* #4	74 %	26 %	80	100 %	0 %	136	97.1
R-558 / 35S-*SDD1* #5	100 %	0 %	45	100 %	0 %	122	87.1
R-558 / 35S-*SDD1* #6	80 %	20 %	70	100 %	0 %	195	139.3
R-558 / 35S-*SDD1* #7	53 %	47 %	131	98 %	2 %	200	142.9
R-558 / 35S-*SDD1* #8	91 %	9 %	68	100 %	0 %	123	87.9
R-558 / 35S-*SDD1* #9	80 %	20 %	90	95.1 %	4.9 %	123	87.9
R-558 / 35S-*SDD1* #10	94 %	6 %	65	100 %	0 %	108	77.1

[a] Stomata separated from other stomata by at least one epidermal cell. [b] Stomata placed in direct contact to at least one other stoma. [c] Number of stomata sampled.

As shown in Table 1, 10 out of 10 and 2 out of 10 transformants harbouring the T-DNAs of the pG-*SDD1* or the p35S-*SDD1* plasmids, respectively, showed a wildtype phenotype on cotyledons with respect to the appearance/absence of clustered stomata. 7 transformants carrying the T-DNA of p35S-*SDD1* showed an intermediate phenotype in cotyledons due to inappropriate expression of the transgene in this organ. In primary leaves, all 10 transformants harboring the T-DNA of pG-*SDD1* and all 10 transformants carrying the T-DNA of p35S-*SDD1* showed a strong reduction in stomatal density and/or the fraction of clustered stomata as compared to the R-558 mutant. These data unequivocally demonstrated the identity of the 2325 bp DNA fragment as the coding region of the *SDD1* gene.

Example 4: Analysis of the *SDD1* nucleotide sequence and SDD1 amino acid sequence

The analysis of the *SDD1* nucleotide and derived amino acid sequences was performed using the GCG 8.1 and BLAST 2.0 computer programs (see: Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711; Altschul et al. 1997, *Nucleic Acids Res.* 25, 3389-3402).

The derived amino acid sequence of *SDD1* shows significant identity / similarity to known members of a specific class of serine proteases, called subtilisins or dibasic processing endoproteases. In subtilisins, four regions form the catalytic triad and the substrate binding site and are most highly conserved among subtilisins, prominent representatives of which are the bacterial SUBTILISIN BPN´ (Wells et al. 1983, Nucleic Acids Res. 11, 7911-7925), the KEX2 of yeast (Mizuno et al. 1988, *Biochem. Biophys. Res. Commun.* 156, 246-254), the human furin/PACE (Wise et al. 1990, *Proc. Natl. Acad. Sci USA* 87, 9378 - 9382) and PC1/PC3 (Seidah et al., 1991, *Mol. Endocrinol.* 5, 111 - 122; Smeekens et al., 1991, *Proc. Natl. Acad. Sci. USA* 88, 340 - 344). Several genes from plants encoding subtilases have been isolated such as CUCUMISIN from *Cucumis melo* (Yamagata et al., 1994, *J. Biol. Chem.* 269, 32725 - 32731), P69 from *Lycopersicon esculentum* (Tornero et al., 1996, *Proc. Natl. Acad. Sci. USA* 93, 6332 - 6337), or AG12 from *Alnus glutinosa*

(Ribeiro et al., 1995, *Plant Cell* 7, 785 - 794). The *in vivo* functions of these plant enzymes, however, are hitherto unknown. In the four conserved regions, SDD1 displays highest sequence similarity to the subtilisins listed above and contains the four characteristic invariant amino acids present in all subtilisins hitherto known (Fig. 5). This unequivocally proves the belonging of SDD1 to this class of endoproteases. The amino acid sequence motif VICAAGNNG within the substrate binding site, however, is unique and distinguishes *SDD1* from all other known subtilisins. The mutation present in the R-558 mutant creates a premature stop codon leading to the formation of a C-terminally truncated protein which lacks the essential S-domain containing the catalytically active serine residue (Fig. 2).

Example 5: Modulation of stomatal density in plants through modulation of *SDD1* expression by genetic engineering

The usefulness of the *SDD1* gene for the creation of plants with various different levels of increased or decreased stomatal densities through modulation of the degree of *SDD1* gene expression was shown by the analysis of further transgenic plants. 4 out of 10 of the transgenic plants carrying the aforementioned (Example 4) T-DNA of p35S-*SDD1* and 1 out of 10 of the transgenic plants carrying the aforementioned (Example 4) T-DNA of pG-*SDD1* showed lower stomatal density than the corresponding wildtype plants analyzed in parallel (Table 1).

Furthermore, *SDD1* antisense inhibition studies were performed: To this end, the plasmid p35S-α*SDD1* was generated which contains an antisense-gene construct called `35S-α*SDD1*´ (Fig. 6). A 2079 bp - fragment (position 74 - 2153 according to the sequence shown in seq. ID 1) of the *SDD1* gene was PCR-amplified and subcloned into the pCR 2.1 - vector (Invitrogen, Leek The Netherlands). Using the flanking Asp718 (3´) and Xbal (5´) restriction sites, the 2 kb *SDD1* - fragment was cut from the pCR 2.1 - vector and inserted into the Asp718 and Xbal sites of the pBIN-AR-Hyg. vector (see example 3), thus placing it in antisense orientation to the CaMV 35S – promoter.

The plasmid p35S-α*SDD1* was introduced into *Agrobacterium tumefaciens* according to Höfgen and Willmitzer (*Nucleic Acids Res.* 16, 9887, 1988) and was

used to generate transgenic *Arabidopsis thaliana* plants through application of the procedure described by Schmidt and Willmitzer (*Plant Cell Rep.* 7, 583-586, 1988). Among the transgenic plants carrying the T-DNA of p35S-α*SDD1* thus generated, individuals with increased stomatal density were obtained.

It was thus demonstrated that through the application of genetic engineering techniques, a gene encoding a subtilisin-like serine protease, can be used to generate plants with various different levels of decreased or increased stomatal densities brought about by the modulation of the expression of said gene.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.
(B) STREET:
(C) CITY: Berlin
(D) STATE: none
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP):

(ii) TITLE OF INVENTION: Means and methods for modulating stomata characteristica in plants

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

SEQ. ID NO: 1

Wild type sequence of SDD1
length: 2328 base pairs
type: nucleic acid
strandedness: double
topology: linear
molecule type: genomic
hypothetical: no
anti-sense: no
original source:
organism: Arabidopsis thaliana
immediate source: library: IGF-BAC library
clone: F20D22
coding sequence: 1 .. 2328

```
   1  atggaaccca aacctttctt tctctgcatt atctttcttc tattttgttc

  51  ttcttcgtca gagatcctgc agaagcagac ttacattgtt cagcttcatc

 101  ctaatagcga aaccgctaaa acctttgcct caaagtttga ttggcatctt

 151  tcttttctcc aagaagcggt tttaggtgtt gaagaagaag aggaagagcc

 201  ttcttctcga cttctctact cctatggctc tgcgattgaa ggatttgctg

 251  ctcagttgac tgaatcagaa gccgagatac tgagatattc acctgaagtt
```

```
 301  gttgcagtga gacctgacca tgttcttcag gttcaaacca cttactctta
 351  caagttcttg ggactcgacg gttttggaaa ctccggtgta tggtctaaat
 401  ctcggtttgg tcaaggcaca attatcggcg tgcttgatac tggagtttgg
 451  cctgaaagtc ctagctttga cgataccgga atgccttcga ttccacggaa
 501  atggaaaggg atttgccaag aaggagaaag tttcagttct tcgagctgta
 551  accggaagct aatcggtgct agattcttca tcagaggaca ccgtgtcgct
 601  aattcaccag aggaatcacc aaacatgcct cgtgaataca tttccgcaag
 651  agattcaacg ggacacggga ctcacaccgc ctcaacagtt ggtggatcct
 701  ctgtttcgat ggcgaatgtt cttggcaatg gagctggtgt ggctcgtggg
 751  atggctcctg gagctcacat tgcagtctat aaagtctgtt ggttcaatgg
 801  ttgttacagc tctgacattc tagcagctat agatgtagcg attcaagata
 851  aagtcgatgt tctttcgctt tcccttggcg gtttccctat tcctttgtat
 901  gatgacacaa tcgccattgg aacattccga gccatggaac gcggtatatc
 951  tgtaatctgt gcagctggta acaacggtcc aatcgaaagc tctgttgcaa
1001  acacagctcc ttgggtctca accattggcg caggcacgct tgatcgaaga
1051  tttcccgctg tggtcagatt agccaacgga aagcttctct atggagagtc
1101  attgtatccg ggaaaaggta taaagaatgc cgggagagag gttgaggtga
1151  tttacgtcac aggaggagat aaaggaagtg agttctgttt gagagggtca
1201  cttccaagag aagaaatccg aggcaaaatg gtgatttgtg atcgcggagt
1251  caatggaaga tcggagaaag gagaagcggt taaagaagct ggaggagttg
1301  caatgatctt agccaataca gagatcaacc aagaagaaga ttctattgac
1351  gttcatctct taccagctac attgattggt tacactgagt cagtccttct
1401  gaaggcttat gttaatgcca cggtgaaacc aaaggcgcgg ataatttttg
1451  gtggtacggt gattgggagg tcacgagcac cggaggtggc tcagttttca
1501  gctcgaggac cgagtttagc caatccttcg atactaaaac cggatatgat
1551  tgctccggga gtcaatatca ttgcggcttg gcctcaaaat ctaggaccaa
1601  ccggacttcc ttatgattca agaagagtta acttcactgt aatgtcagga
1651  acttcaatgt cttgtccaca tgttagcgga atcactgctc ttatccggtc
1701  tgcatacccg aactggtctc cagctgcaat caaatccgca ttgatgacaa
1751  cagcggattt gtacgatcgt caagggaaag cgataaagga tggtaacaaa
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1801 ccagccggtg tgtttgcgat tggagcaggg catgtgaatc cgcaaaaggc

1851 gataaacccg ggattggttt acaacattca accagtggat tacataactt

1901 acctctgcac tcttggattc acaagatcag atattttagc aatcactcat

1951 aagaacgtga gctgcaatgg aatattgcgg aaaaacccgg gttttagtct

2001 caattacccg tcgatagccg tgattttcaa acgtggcaag actacggaga

2051 tgatcacaag gcgtgtcact aacgttggga gtcctaactc gatatactca

2101 gtgaatgtca aggctccaga ggggatcaaa gttattgtca atcctaagag

2151 acttgtgttc aaacacgtgg atcagacgct gagctataga gtatggtttg

2201 tattgaagaa gaaaaacaga ggagggaagg tggctagctt tgcacaaggg

2251 cagttgactt gggtcaactc tcataatctg atgcagcgag ttagaagtcc

2301 aatctctgta accttgaaga ctaactga

SEQ. ID NO: 2

mutant sequence of *sdd1* (mutated nucleotide underlined)
length: 2328 base pairs
type: nucleic acid
strandedness: double
topology: linear
molecule type: genomic
hypothetical: no
anti-sense: no
original source:
organism: *Arabidopsis thaliana*
immediate source: *Arabidopsis thaliana* mutant R-558
coding sequence: 1 … 2328

1 atggaaccca aacctttctt tctctgcatt atctttcttc tattttgttc

51 ttcttcgtca gagatcctgc agaagcagac ttacattgtt cagcttcatc

101 ctaatagcga aaccgctaaa acctttgcct caaagtttga ttggcatctt

151 tcttttctcc aagaagcggt tttaggtgtt gaagaagaag aggaagagcc

201 ttcttctcga cttctctact cctatggctc tgcgattgaa ggatttgctg

251 ctcagttgac tgaatcagaa gccgagatac tgagatattc acctgaagtt

301 gttgcagtga gacctgacca tgttcttcag gttcaaacca cttactctta

351 caagttcttg ggactcgacg gttttggaaa ctccggtgta tggtctaaat

401 ctcggtttgg tcaaggcaca attatcggcg tgcttgatac tggagtttgg

451 cctgaaagtc ctagctttga cgataccgga atgccttcga ttccacggaa

501 atggaaaggg atttgccaag aaggagaaag tttcagttct tcgagctgta

551 accggaagct aatcggtgct agattcttca tcagaggaca ccgtgtcgct

601 aattcaccag aggaatcacc aaacatgcct cgtgaataca tttccgcaag

651 agattcaacg ggacacggga ctcacaccgc ctcaacagtt ggtggatcct

701 ctgtttcgat ggcgaatgtt cttggcaatg gagctggtgt ggctcgtggg

751 atggctcctg gagctcacat tgcagtctat aaagtctgtt ggttcaatgg

801 ttgttacagc tctgacattc tagcagctat agatgtagcg attcaagata

851 aagtcgatgt tctttcgctt tcccttggcg gtttccctat tcctttgtat

901 gatgacacaa tcgccattgg aacattccga gccatggaac gcggtatatc

951 tgtaatctgt gcagctggta acaacggtcc aatcgaaagc tctgttgcaa

1001 acacagctcc ttgggtctca accattggcg caggcacgct tgatcgaaga

1051 tttcccgctg tggtcagatt agccaacgga aagcttctct atggagagtc

1101 attgtatccg ggaaaaggta taaagaatgc cgggagagag gttgaggtga

1151 tttacgtcac aggaggagat aaaggaagtg agttctgttt gagagggtca

1201 cttccaagag aagaaatccg aggcaaaatg gtgatttgtg atcgcggagt

1251 caatggaaga tcggagaaag gagaagcggt taaagaagct ggaggagttg

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1701 tgcatacccg aactggtctc cagctgcaat caaatccgca ttgatgacaa

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1801 ccagccggtg tgtttgcgat tggagcaggg catgtgaatc cgcaaaaggc

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2001  caattacccg tcgatagccg tgattttcaa acgtggcaag actacggaga
2051  tgatcacaag gcgtgtcact aacgttggga gtcctaactc gatatactca
2101  gtgaatgtca aggctccaga ggggatcaaa gttattgtca atcctaagag
2151  acttgtgttc aaacacgtgg atcagacgct gagctataga gtatggtttg
2201  tattgaagaa gaaaaacaga ggagggaagg tggctagctt tgcacaaggg
2251  cagttgactt gggtcaactc tcataatctg atgcagcgag ttagaagtcc
2301  aatctctgta accttgaaga ctaactga
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SEQ. ID NO: 3

Wild type sequence of SDD1
length: 775 amino acids
type: amino acid
topology: linear
molecule type: protein

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 101  VAVRPDHVLQ VQTTYSYKFL GLDGFGNSGV WSKSRFGQGT IIGVLDTGVW
 151  PESPSFDDTG MPSIPRKWKG ICQEGESFSS SSCNRKLIGA RFFIRGHRVA
 201  NSPEESPNMP REYISARDST GHGTHTASTV GGSSVSMANV LGNGAGVARG
 251  MAPGAHIAVY KVCWFNGCYS SDILAAIDVA IQDKVDVLSL SLGGFPIPLY
 301  DDTIAIGTFR AMERGISVIC AAGNNGPIES SVANTAPWVS TIGAGTLDRR
 351  FPAVVRLANG KLLYGESLYP GKGIKNAGRE VEVIYVTGGD KGSEFCLRGS
 401  LPREEIRGKM VICDRGVNGR SEKGEAVKEA GGVAMILANT EINQEEDSID
 451  VHLLPATLIG YTESVLLKAY VNATVKPKAR IIFGGTVIGR SRAPEVAQFS
 501  ARGPSLANPS ILKPDMIAPG VNIIAAWPQN LGPTGLPYDS RRVNFTVMSG
 551  TSMSCPHVSG ITALIRSAYP NWSPAAIKSA LMTTADLYDR QGKAIKDGNK
 601  PAGVFAIGAG HVNPQKAINP GLVYNIQPVD YITYLCTLGF TRSDILAITH
 651  KNVSCNGILR KNPGFSLNYP SIAVIFKRGK TTEMITRRVT NVGSPNSIYS
 701  VNVKAPEGIK VIVNPKRLVF KHVDQTLSYR VWFVLKKKNR GGKVASFAQG
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751 QLTWVNSHNL MQRVRSPISV TLKTN*

SEQ. ID NO: 4

mutant sequence of *sdd1*
length: 491 amino acids
type: amino acid
topology: linear
molecule type: protein

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51 SFLQEAVLGV EEEEEEPSSR LLYSYGSAIE GFAAQLTESE AEILRYSPEV

101 VAVRPDHVLQ VQTTYSYKFL GLDGFGNSGV WSKSRFGQGT IIGVLDTGVW

151 PESPSFDDTG MPSIPRKWKG ICQEGESFSS SSCNRKLIGA RFFIRGHRVA

201 NSPEESPNMP REYISARDST GHGTHTASTV GGSSVSMANV LGNGAGVARG

251 MAPGAHIAVY KVCWFNGCYS SDILAAIDVA IQDKVDVLSL SLGGFPIPLY

301 DDTIAIGTFR AMERGISVIC AAGNNGPIES SVANTAPWVS TIGAGTLDRR

351 FPAVVRLANG KLLYGESLYP GKGIKNAGRE VEVIYVTGGD KGSEFCLRGS

401 LPREEIRGKM VICDRGVNGR SEKGEAVKEA GGVAMILANT EINQEEDSID

451 VHLLPATLIG YTESVLLKAY VNATVKPKAR IIFGGTVIGR S*

SEQ. ID NO: 5

Sequence of SDD1 promoter
length: 1970 base pairs
type: nucleic acid
strandedness: double
topology: linear
molecule type: genomic
hypothetical: no
anti-sense: no
original source:
organism: Arabidopsis thaliana
immediate source: library: IGF-BAC library
clone: F20D22
coding sequence: -

```
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 101  cgtagtcgcc ttgggattat acctcaagat cctacattat ttgaaggaac
 151  aatccgagca aatcttgacc cacttgaaga acattcagat gataaaatct
 201  gggaggtatc tataaatatg ttgtttgata ctcttatctt gtttatgttt
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 651  ccggatacca actgttatag acagtgacct agttttggtt ctcagcgacg
 701  gttagtctca tacaaattaa aaacatggat ctttcttcat attactcgtc
 751  gtcttttggg agaattcaat gtttatgttt atggtttgtt gcaggtagag
 801  tagcagagtt tgatactcct gcacggctat tagaagacaa atcatcgatg
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1301  gtacgacgtc gttttgttgt gacgtagtac ccgatgacaa aacgaggcgt
1351  gttgagcgtg cgtgtataaa tgcacgtagt gaacgaacac taaccacgct
1401  gctgcattta attcctttct caacggtcgt attttcctat tcaaggcttt
1451  aactaagttt aatagtatgt ttttaaaaaa atacatatat ttggccaatg
1501  gttgattatt aagttattgt taaatgaatt ttctttggct tggtaaaact
1551  tcaatggaaa ccaaataaat ttcaaaatct tttgttgctg aaaaaggcta
1601  cacaaactac tatacgcaat aaaacctaac cataacatct cgtgaacaag
1651  gaaaaaaaaa ggaaaaacag agacacagag acaaggcaga caaaaaagcc
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1801  ctcgtgaggc agagctctta ctcttcacga gcttcatcat cttcttcctt
1851  agactccaaa tcccaggttt tgaattctct tcattcttct ttaaataccc
1901  tattcttctt cttctctaaa accatgcact tcaactacac taaaccaact
1951  ccttttttta actctctcca
```

Claims

1. A recombinant DNA molecule comprising:

(i) a nucleic acid molecule encoding a subtilisin-like serine protease or encoding a biologically active fragment of such a protein, selected from the group consisting of

(a) nucleic acid molecules comprising a nucleotide sequence encoding a protein comprising the amino acid sequence as given in SEQ ID NO: 3;

(b) nucleic acid molecules comprising a nucleotide sequence as given in SEQ ID NO: 1;

(c) nucleic acid molecules encoding a protein comprising at least the D region, H region, substrate binding site and/or S region of the subtilisin-like serine protease encoded by a nucleic acid molecule of (a) or (b); or

(d) nucleic acid molecules hybridizing with the complementary strand of a nucleic acid molecule as defined in any one of (a) to (c);

(e) nucleic acid molecules encoding a protein the amino acid sequence of which is at least 50% identical to the amino acid sequence encoded by a nucleic acid molecule of any one of (a) to (c);

(f) nucleic acid molecules, the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of a nucleic acid molecule as defined in any one of (a) to (e); or

(ii) a nucleic acid molecule encoding a mutant non-active or a hyper-active form of or an antibody against the subtilisin-like serine protease encoded by a nucleic acid molecule of (i); or

(iii) a nucleic acid molecule which specifically hybridizes with a nucleic acid molecule of (i) or the complementary strand thereof.

2. The recombinant DNA molecule of claim 1 wherein the nucleic acid molecule is DNA, cDNA, genomic DNA or synthetically synthesized DNA.

3. The recombinant DNA molecule of claim 1 wherein the nucleic acid molecule is derived from a plant, preferably Arabidopsis.

4. The recombinant DNA molecule of any one of claims 1 to 3 wherein said nucleic acid molecule is operably linked to regulatory elements allowing the expression of the nucleic acid molecule in plants.

5. A vector comprising a recombinant DNA molecule of any one of claims 1 to 4.

6. A host cell containing a vector of claim 5 or a recombinant DNA molecule of any one of claims 1 to 4.

7. A method for the production of transgenic plants with altered stomata characteristica compared to wild type plants comprising the introduction of a recombinant DNA molecule of any one of claims 1 to 4 or the vector of claim 5 into the genome of a plant, plant cell or plant tissue.

8. A transgenic plant cell comprising stably integrated into the genome a recombinant DNA molecule of any one of claims 1 to 4 or a vector of claim 5 or obtainable according to the method of claim 7, wherein the expression of the nucleic acid molecule results in an increased expression or activity of subtilisin-like serine proteases in transgenic plants compared to wild type plants.

9. A transgenic plant or a plant tissue comprising plant cells of claim 8.

10. The transgenic plant of claim 9 which displays a decreased stomata density, lower conductance of stomata and/or wherein the water consumption is lowered compared to wild type plants.

11. A transgenic plant cell which contains stably integrated into the genome a recombinant DNA molecule of any one of claims 1 to 4 or part thereof, a vector of claim 5 or obtainable according to the method of claim 7, wherein the presence, transcription and/or expression of the nucleic acid molecule or part thereof leads to reduction of the synthesis or the activity of subtilisin-like serine proteases in transgenic plants compared to wild type plants.

12. The plant cell of claim 11, wherein the reduction is achieved by an antisense, sense, ribozyme, co-suppression and/or dominant mutant effect.

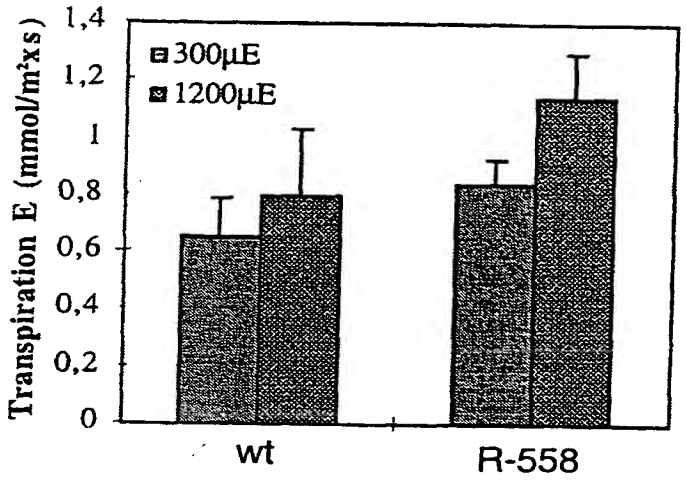
13. A transgenic plant or plant tissue comprising the plant cells of claim 11 or 12.

14. The transgenic plant of claim 13 which displays increased stomatal density, higher conductance of stomata and/or increased content of sugars and/or protein in plant leaves compared to wild type plants.

15. Harvestable parts or propagation material of plants of any one of claims 9, 10, 13 or 14 comprising plant cells of claim 8, 11 or 12.

16. A kit comprising a recombinant DNA molecule of any one of claims 1 to 4 or a vector of claim 5.

17. Use of a nucleic acid molecule encoding or regulating the expression of a subtilisin-like serine protease or a nucleic acid molecule hybridizing with such a nucleic acid molecule, a nucleic acid molecule as defined in any one of claims 1 to 4, a recombinant DNA molecule of any one of claims 1 to 4, or a vector of claim 5 for the production of plants with improved fresh and dry weight, for enhancing the content of sugars and/or protein in plant leaves for the production of plants with reduced leaf temperatures or with reduced water loss and lower water consumption, for the modulation (enhancement) of CO_2 uptake into and H_2O release from leaves, for sustained photosynthesis under high intensity conditions or for the improvement of disease resistance of plants.



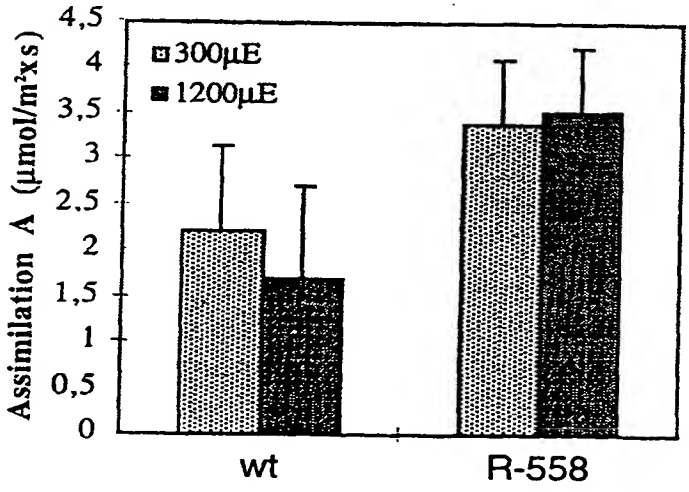


Figure 1

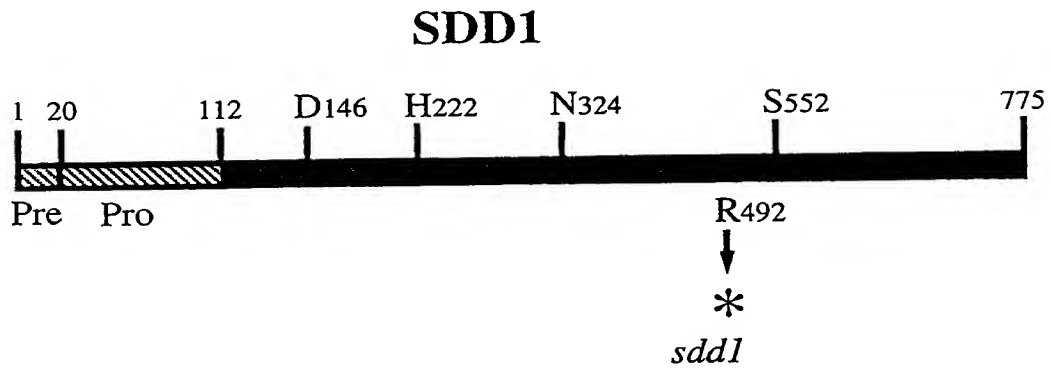
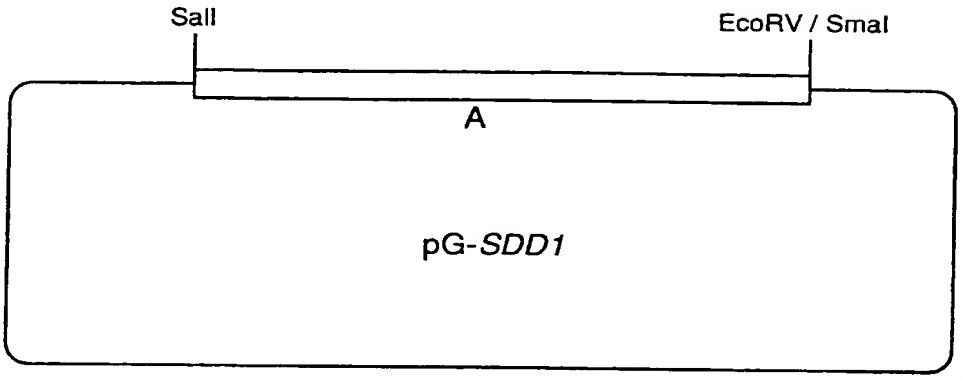


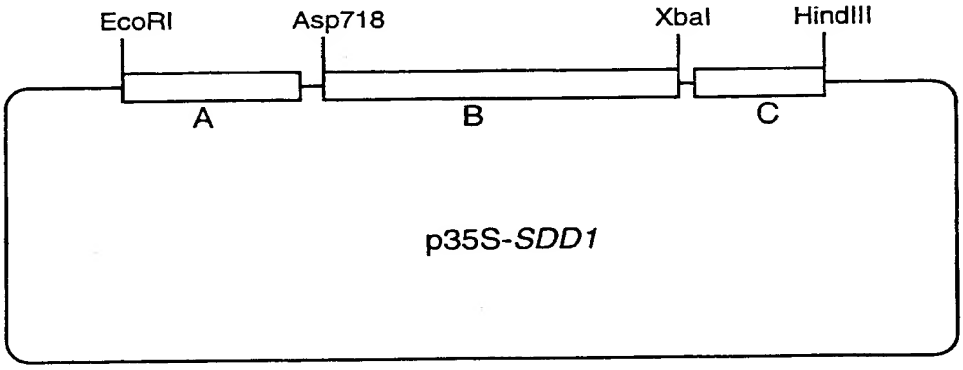
Figure 2



Fragment A: 7067 bp SalI - EcoRV subfragment of the BAC IGF20D22 that includes the 2328 bp coding region of *SDD1* in addition to 2 kb upstream DNA (promotor) and 2.8 kb downstream DNA was inserted into the SalI and SmaI sites of the vector pBIB-Hyg.

Vector: pBIB-Hyg (Becker, 1990, *Nucleic Acids Res.* 18, 203).

Figure 3



Fragment A: 35S promoter of the Cauliflower Mosaic Virus; (Gardner et al., 1981,*Nucleic Acids Res.* 9, 2871-2888)

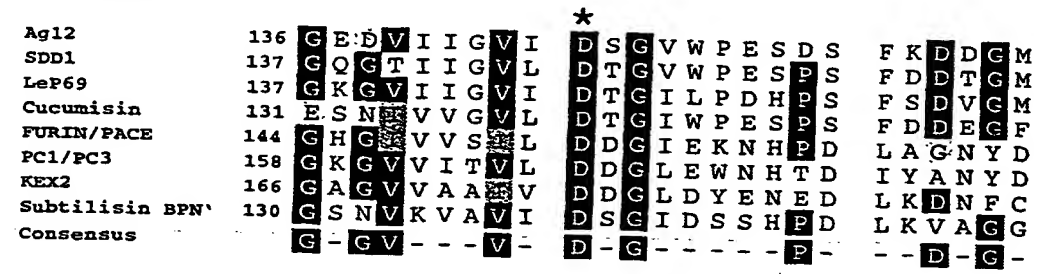
Fragment B: 2328 nucleotides coding region of the *SDD1* gene (seq. ID no. 1)

Fragment C: polyadenylation signal of the gene 3 from the T-DNA of the Ti-plasmid pTi ACH 5 (Gielen et al., 1984, *EMBO J.* 3, 835 – 846)

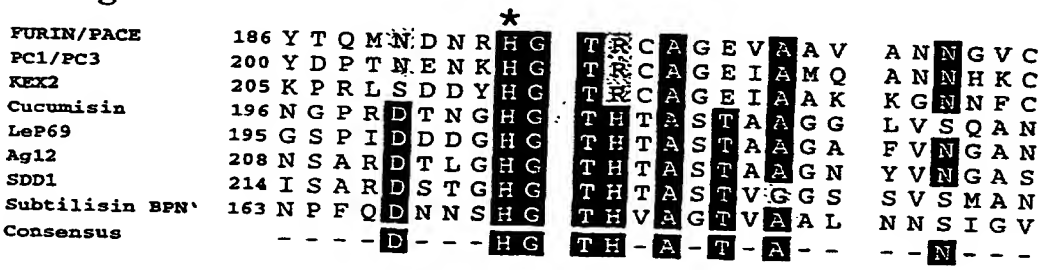
Vector: pBIB-Hyg (Becker, 1990, *Nucleic Acids Res.* 18, 203).

Figure 4

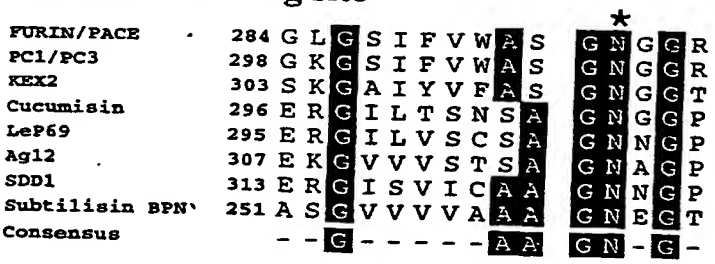
D region



H region



substrate binding site



S region

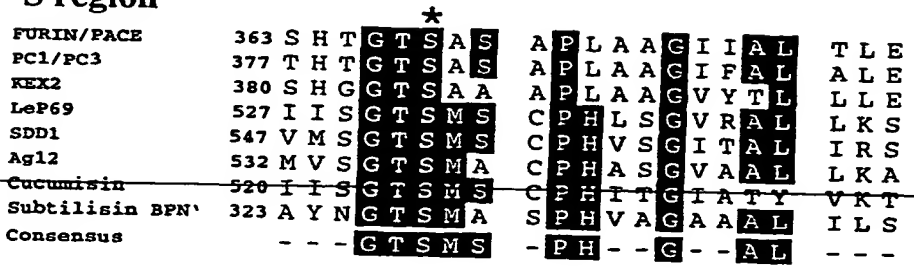
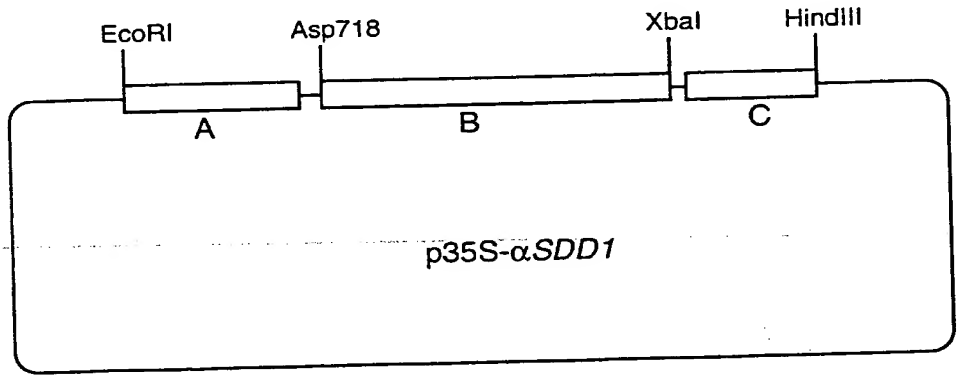


Figure 5



Fragment A: 35S promoter of the Cauliflower Mosaic Virus; (Gardner et al., 1981,*Nucleic Acids Res.* 9, 2871-2888)

Fragment B: 2079 bp - fragment (position 74 - 2153 according to the sequence shown in seq. ID 1) of the *SDD1* gene inserted in antisense orientation to the 35S promoter.

Fragment C: polyadenylation signal of the gene 3 from the T-DNA of the Ti-plasmid pTi ACH 5 (Gielen et al., 1984, *EMBO J.* 3, 835 – 846)

Vector: pBIB-Hyg (Becker, 1990, *Nucleic Acids Res.* 18, 203).

Figure 6

Abstract

Described is the use of nucleic acid molecules encoding subtilisin-like serine proteases and the modulation of the corresponding genes for the production of transgenic plants with altered stomata characteristics. Provided are recombinant DNA molecules comprising such nucleic acid molecules and complements thereof; wherein said nucleic acid molecule(s) are operably linked to regulatory elements allowing the expression of the nucleic acid molecule(s) in plants. Also provided are vectors comprising said recombinant DNA molecules as well as plant cells, plant tissues and plants transformed therewith. In addition, the use of the aforementioned nucleic acid molecules, recombinant DNA molecules and vectors in plant cell and tissue culture, plant breeding and/or agriculture is described, in particular for the production of plants with improved phenotypes.